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A domain directly C-terminal to the major homology region of human immunodeficiency type 1 capsid protein plays a crucial role in directing both virus assembly and incorporation of Gag–Pol

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Abstract

We demonstrate here that a deletion of 14 amino acid residues directly C-terminal to the major homology region (MHR) of the HIV-1 capsid (CA) in Gag–Pol markedly affects the incorporation of Gag–Pol into virions. The 14-amino acid deletion also significantly impaired virus assembly. In agreement with previous reports, mutations at the very C-terminus of CA resulted in a remarkable reduction in virus production. However, HIV-1 Gag–Pol precursors containing the C-terminal CA mutation were still capable of being incorporated into virions at a level of about 50% that of the wild-type. These results suggest that the domain immediately C-terminal to the MHR is functionally involved in Gag–Gag and Gag/Gag–Pol interaction, and this supports the notion that Gag or Gag–Pol mutants blocked in assembly into virus particles can be rescued into virions provided they retain the domains that are able to interact with the Gag precursor. An HIV-1 Gag–Pol deletion mutant retaining a minimal sequence consisting of the MHR and the adjacent CA-SP1 was efficiently incorporated into virions. Analysis by immunofluorescence staining indicated that the subcellular localization patterns shown by the Gag–Pol mutants were not fully compatible with their efficiency in being incorporated into virions, suggesting that the ability of Gag–Pol mutants to be incorporated into virions largely depends on their interactions with the Gag precursor.

Keywords: Human immunodeficiency virus; Gag assembly; Gag–Pol incorporation; Major homology region (MHR); CA-SP1

Introduction

The *gag* gene of all retroviruses, including human immunodeficiency virus (HIV), encodes the viral structural protein Gag (Hunter, 1994; Swanstrom and Wills, 1997). In HIV, Gag is synthesized as a polyprotein precursor, $Pr55^{gag}$. During or soon after virus budding, $Pr55^{gag}$ is cleaved by the *pol*-encoded protease (PR) into several distinct products, namely matrix (MA; p17), capsid (CA; p24), nucleocapsid (NC; p7), and the C-terminal p6 domain (Freed, 1998; Henderson et al., 1992; Kaplan et al., 1994; Leis et al., 1988; Mervis et al., 1988; Swanstrom and Wills, 1997). In addition, two small cleavage products termed SP1 and SP2 separate NC from CA and from p6, respectively. The *pol* gene, which partially overlaps with the *gag* gene, encodes PR, reverse transcriptase (RT), RNase H, and intergrase (IN). Pol is translated as a polyprotein precursor, $Pr160^{gag–pol}$, by a −1 ribosomal frameshift mechanism (Jacks et al., 1988). Within $Pr160^{gag–pol}$, the $p6^{gag}$ is truncated and replaced by a transframe polypeptide (TF) termed the p6* or $p6^{pol}$ domain (Partin et al., 1990). The ribosomal frameshift event occurs at a frequency of about 5–10%, resulting in expression of $Pr55^{gag}$ and $Pr160^{gag–pol}$ at a ratio of between 20:1 and 10:1 (Jacks et al., 1988). The maintenance of the lower expression level for Gag–Pol or Gag–PR is critical for virus particle production, as Gag–Pol that is overexpressed artificially can block or significantly reduce the production of virus particles (Arrigo and Huffman, 1995; Burstein et al., 1991; Chiu et al., 2002; Krausslich, 1991; Park and Morrow, 1991; Wang et al., 2000). How the HIV-1 PR is

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activated to mediate the processing of virus particles is still unclear. It is thought that activation of HIV-1 PR is initiated or promoted through the formation of Gag–Pol dimers. The fully functional PR, normally as a homodimer, then cleaves the Gag–Pol and Gag precursors to yield the mature *gag* and *pol* gene products (Navia and McKeever, 1990). The PR-mediated virus maturation process is not required for virus assembly and budding, but is essential for virus infectivity (Chen et al., 1997; Gottlinger et al., 1989; Kohl et al., 1988; Peng et al., 1989; Rose et al., 1995).

For most retroviruses including HIV, expression of *gag* alone is sufficient for virus particle assembly and budding. Genetic studies of retroviral Gag have defined three major domains; these are the membrane-binding domain (M), interaction domain (I), and the late (L) domain, to which specific functional roles in the process of virus assembly and budding have been ascribed. In HIV-1 the L domain is located in the C-terminal of $p6^{gag}$, which contains a PT/SAP motif required for efficient virus budding (Freed, 2002). The HIV-1 M domain contains a myristic acid moiety and a cluster of basic residues at the N-terminus of the MA region. These are required for Gag membrane association and plasma membrane targeting (Bryant and Ratner, 1990; Facke et al., 1993; Ono and Freed, 1999; Ono et al., 2000; Pal et al., 1990). The I domain within the NC facilitates virus assembly by promoting Gag–Gag interactions (Derdowski et al., 2004; Lee et al., 1999; Sandefur et al., 2000; Zhang et al., 1998). Extensive genetic analyses have suggested that the C-terminal domain of CA and the adjacent SP1 are critical for Gag multimerization and virus assembly (Abdurahman et al., 2004; Accola et al., 1998; Ganser-Pornillos et al., 2004; Liang et al., 2002, 2003; Melamed et al., 2004; Morikawa et al., 2000; Reicin et al., 1995; Scarlata and Carter, 2003; von Schwedler et al., 2003; Wang et al., 1998). In vitro studies have also suggested that the Gag oligomerization domain is contained within the C-terminal CA sequence (Ganser-Pornillos et al., 2004). It has been shown that HIV-1 *gag* deletion mutants retaining the N-terminal myristylation signal and the C-terminal Gag domain can still direct virus particle assembly and budding (Accola et al., 2000; Borsetti et al., 1998). This suggests that the C-terminal CA and the adjacent SP1 and NC regions are the most important determinants of Gag oligomerization and assembly.

HIV-1 Gag–Pol is generally thought to be incorporated into virus particles via interactions with the assembling Gag, by virtue of its N-terminal Gag domain. Genetic studies involving coexpression of Gag–Pol deletion mutants with a $Pr55^{gag}$-expression plasmid have shown that the C-terminal domain of Gag in Gag–Pol is the most important region for its incorporation into virus particles (Chiu et al., 2002; Srinivasakumar et al., 1995). It is conceivable that HIV-1 Gag–Pol mutants lacking the *gag* coding sequence involved in Gag oligomerization may be defective in incorporation into virions. In this study, we identified a region immediately C-terminal to the MHR that significantly affected both virus assembly and the incorporation of Gag–Pol into virions. Additionally, we defined a minimal sequence, consisting of the MHR and the adjacent CA-SP1 sequence that is capable of directing the incorporation of Gag–Pol into virions.

Results

The level of incorporated pol gene products from the Gag–Pol mutants did not reflect the efficiency of virus particle processing by Gag–Pol mutants

To define which regions within the C-terminal *gag* sequence of $Pr160^{gag-pol}$ are able to affect the incorporation of $Pr160^{gag-pol}$ into virus particles, a series of $Pr160^{gag-pol}$ mutant constructs with various deletions in the C-terminal *gag* coding sequence was constructed. As illustrated in Fig. 1 and Table 1, constructs Δ310–323 and Δ322–338, respectively, contain a deletion of 14 and 17 residues downstream of the MHR domain. Δ339–365 has a deleted sequence including the very last 25 C-terminal residues of the CA domain and the first two residues of SP1; both Δ374–378 and Δ368–378 have the SP1 sequence partially or almost deleted, respectively; Δ385–429 has the NC domain deleted. As described previously, Δ16–429 has the *gag* coding sequence almost deleted (Chiu et al., 2002). Further C-terminal deletions extending to include the $p6^{pol}$ and/or the PR domain yielded constructs Δ16-PR and Δ16-RT, respectively. HIV-1 Gag–Pol lacking the MHR (Δ281–308), or the N-terminal two-thirds of the *gag* coding sequence but retaining the myristylation signal (Δ16–282), has been shown to be capable of being incorporated into virions (Chiu et al., 2002, 2004). These two mutations were included as controls in this study. Double-mutation constructs were made by introducing the Δ16–282 deletion into the above Gag–Pol constructs, to give constructs Δ16–282/310–323, Δ16–282/339–365, Δ16–282/368–378, Δ16–282/374–378, and Δ16–282/385–429.

To test the effect of the deletion mutations on the incorporation of Gag–Pol into virus particles, wt GPfs or each of the GPfs mutants (except the PR-defective mutant Δ16-RT) were cotransfected with the $Pr55^{gag}$-expressing plasmid pGAG into 293T cells. The ability of each of the Gag–Pol deletion mutants to be incorporated into virus particles and to process Gag particles was analyzed by Western immunoblot. The results shown in Fig. 2 suggest that all the Gag–Pol mutants except Δ16–429 and Δ16-PR could be incorporated and could process Gag particles, as the virus-associated mature $p24^{gag}$ could be readily detected (lanes 2–7 and 11–16). The relatively lower level of virus-associated RT observed with Δ16–429 and Δ16–PR may reflect their severe defects in both virus particle incorporation and processing. However, the level of virus-associated RT shown by the mutants did not completely correlate with the efficiency of their virus particle processing. For example, the Δ16–282 and the double mutants derived from it all showed a Gag processing profile similar to that of the wt; however, Δ16–282 and Δ16–282/374–378 were able to and the other three double mutants were unable to produce substantial amounts of virus-associated RT (lanes 10–15). This suggests that some of the Gag–Pol deletion mutations may have

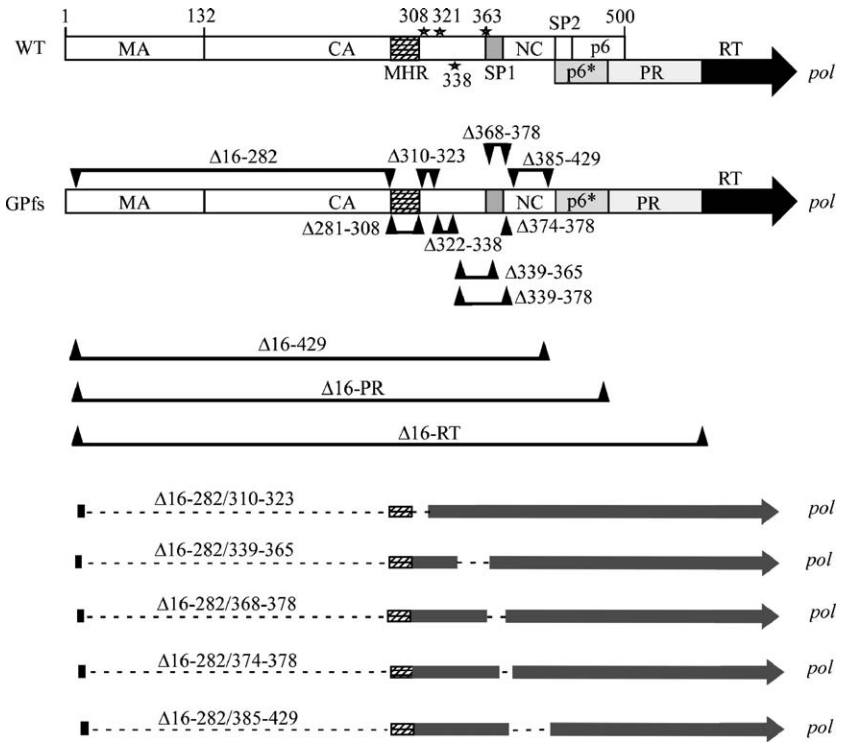

Fig. 1. Schematic representations of HIV-1 Pr160$^{gag–pol}$ mutants. Wild-type (WT) Gag protein domains, major homology region (MHR), and the *pol*-encoded p6*, PR, and RT domains are indicated. All of the Gag–Pol mutants are derived from the plasmid GPfs, which were constructed by placing *gag* and *pol* in the same open reading frame via a five-T nucleotide deletion in the overlap region of *gag–pol*. The numbers indicate the position of the amino acid residue of the HIV-1 Gag precursor, and are used to denote each of the mutant constructs with either a linker insertion (asterisks) or a deletion mutation in the *gag–pol* coding sequence. The boundaries of the deleted *gag–pol* sequences are indicated (solid lines and arrowheads). Mutations with deletion of the sequence from the Gag residue 16 to the N-terminus of the PR and RT domain are designated as Δ16-PR and Δ16-RT, respectively. Dashed lines in the double mutant constructs indicate deleted regions. The sequences in the mutated region are given in Table 1.

significantly affected PR activity, and that a minimal amount of incorporated Gag–Pol may well provide sufficient PR activity for Gag processing.

HIV-1 Gag–Pol precursors lacking 14 amino acid residues directly C-terminal to the MHR were not capable of being incorporated into virions, while Gag–Pol precursors retaining a minimal sequence consisting of the MHR and the adjacent CA-SP1 were not blocked

Since both the level of virus particle production and the process of Gag–Pol incorporation into virions can be influenced by the PR activity expressed in parallel, it might be possible to assess the level of virus-associated Gag–Pol more accurately if PR was inactivated. To do so, a PR-defective mutation was introduced into the wt and mutant GPfs constructs, and each of the resultant PR-defective constructs was introduced by cotransfection with pGAG at a DNA ratio of 1:1. The results shown in Fig. 3 indicate that deletions of the MHR alone (Δ281–308) or the sequence upstream of MHR (Δ16–282) had only mild to moderate effects on Gag–Pol incorporation (Fig. 3A, lane 2, and Fig. 3B, lane 3), which is consistent with a previous report (Chiu et al., 2002). In contrast, a deletion of the 14 residues immediately C-terminal to the MHR (Δ310–323) dramatically blocked Gag–Pol incorporation (Fig. 3A, lane 3). The Δ322–338 deletion showed a moderate effect on Gag–Pol incorporation (Fig. 3A, lane 10). Surprisingly, the Δ339–365 construct, with a deletion of the sequence shown to be crucial for Gag oligomerization and virus assembly (Abdurahman et al., 2004; Liang et al., 2003; Melamed et al., 2004; Ganser-Pornillos et al., 2004) had only a moderate effect on the incorporation of Gag–Pol into virions (lane 4). Constructs Δ368–378 (lane 5), Δ374–378 (lane 6), and Δ385–429 (data not shown) all produced a level of virus-associated Gag–Pol comparable to that of wt, suggesting that mutations in the SP1 domain and the adjacent NC domain of Gag–Pol have no major effects on the incorporation of Gag–Pol into virions. Since the SP1 domain and the CA–SP1 boundary sequence have both been shown to play a crucial role in the process of Gag multimerization (Accola et al., 1998; Liang et al., 2002, 2003; Guo et al., 2005), it would therefore be of interest to test whether removal of the C-terminus of CA and the adjacent SP1 from the HIV-1 Gag–Pol can affect the incorporation of Gag–Pol into virus particles. The results shown in Fig. 3C suggest that HIV-1Gag–Pol with a deletion of the C-terminus and the adjacent SP1 (Δ339–378) could still be incorporated into virions at a level of efficiency comparable to that of wt.

Intriguingly, the double mutations Δ16–282/339–365 or Δ16–282/368–378 significantly affected the incorporation of Gag–Pol into virus particles, while Δ16–282/374–378 or Δ16–282/385–429 had only modest effects on Gag–Pol incorporation (Fig. 3B, lanes 5–8). Deletions of the coding

Table 1
Nucleotide sequence and altered amino acid residues at the juncture of mutated regions

Constructs	Sequences in mutated regions[a]							
Δ16–282	nt 830–5′	cga	tgg	atc	ctg	gac	ata	3′-nt 1644
		R	W	**I**	L	D	I	
Δ281–308	nt 1622-5′	cct	acc	agg	atc	cga	gct	3′-nt 1716
		P	T	**R**	**I**	**R**	A	
*in*308	nt 1709-5′	caa	gct	cgg	atc	cga	gct	3′-nt 1716
		Q	**A**	**R**	**I**	**R**	A	
*in*321	nt 1745-5′	acc	ttg	tgg	atc	caa	aat	3′-nt 1764
		T	L	**W**	**I**	Q	N	
Δ310–323	nt 1709-5′	caa	gct	cgg	atc	caa	aat	3′-nt 1764
		Q	A	**R**	**I**	Q	N	
*in*338	nt1793-5′	gca	ttg	ggg	atc	cca	gcg	3′-nt 1809
		A	L	G	**I**	P	A	
Δ322–338	nt 1745-5′	acc	ttg	tgg	atc	cca	gcg	3′-nt 1809
		T	L	**W**	**I**	P	A	
*in*363	nt 1873-5′	gtt	tgg	atc	caa	gca	atg	3′-nt 1890
		V	**W**	**I**	**Q**	A	M	
Δ339–365	nt 1796-5′	ttg	ggg	atc	caa	gca	atg	3′-nt 1890
		L	G	**I**	**Q**	A	M	
Δ368–378	nt 1880-5′	gaa	gca	agg	atc	cag	aga	3′-nt 1929
		E	A	**R**	**I**	Q	R	
Δ339–378	nt 1793-5′	gca	ttg	ggg	atc	cag	aga	3′-nt 1929
		A	L	G	**I**	**Q**	R	
Δ374–378	nt 1901-5′	aat	tca	ggg	atc	cag	aga	3′-nt 1929
		N	S	**G**	**I**	Q	R	
Δ385–429	nt 1931-5′	aat	ttt	agg	atc	cag	gct	3′-nt 2082
		N	F	R	**I**	Q	A	
Δ16–429	nt 830-5′	cga	tgg	atc	cag	gct	aat	3′-nt 2085
		R	W	**I**	Q	A	N	
Δ16-PR	nt 830-5′	cga	tgg	atc	cac	ttc	cct	3′-nt 2255
		R	W	**I**	**H**	F	P	
Δ16-RT	nt 830-5′	cga	tgg	atc	cat	ttt	ccc	3′-nt 2552
		R	W	**I**	**H**	F	P	

[a] Inserted or altered amino acid residues are shown in boldface.

sequence upstream of Pol or RT (Δ16–429, Δ16-PR, and Δ16-RT) also significantly impaired the incorporation of Gag–Pol (Fig. 3A, lanes 7–9). These results suggest that the MHR and the adjacent C-terminal CA–SP1 sequence constitute a minimal sequence required for efficient incorporation of Gag–Pol into virions.

Indirect immunofluorescence studies of the Gag–Pol deletion mutants

It is possible that the mutations may have affected Gag–Pol transport and consequently influenced the incorporation of Gag–Pol into virions. To test this possibility, 293T cells were transfected with the wt GPfs or each of the mutant GPfs constructs, and the intracellular location of Gag–Pol was revealed by indirect immunofluorescence. As shown in Fig. 4A, the wt Gag–Pol precursor proteins were detected throughout the cytoplasm of transfected cells and showed a heterogeneous cytoplasmic staining and a clear perinuclear ring. Cells expressing Δ281–308 (B), Δ310–323 (C),Δ322–338 (D), Δ339–365 (E), Δ368–378 (F), Δ374–378 (G), Δ385–429 (H), Δ16–429 (I), Δ16–282 (L) or each of the Δ16–282 double mutants (M–Q) showed a immunofluorescence staining pattern hardly distinguishable from that of wt. In contrast, about 90% of the Δ16-PR or Δ16-RT transfectants displayed fluorescence staining that was asymmetrically enriched for Gag–Pol in the perinuclear area (Figs. 4J and K). This suggests that appropriate cellular transport of Gag–Pol may have been impaired by the two mutations and this may contribute in part to the severe defect that these two mutants show during incorporation into virus particles. However, most of the mutants were defective in particle incorporation despite the fact that they demonstrated a subcellular localization pattern similar to that of the wt (Table 2). These results suggest that the ability of Gag–Pol mutants to be incorporated into virions is largely dependent on whether the mutations have affected the Gag/Gag–Pol interactions.

Mutations in the region immediately C-terminal to the MHR of CA markedly reduce HIV-1 particle production

Since the Δ310–323 mutation in Gag–Pol markedly affected the interaction of Gag–Pol with $Pr55^{gag}$, it is quite possible that this mutation may also affect Gag particle assembly. To test this possibility, the deletion mutations Δ310–323, Δ322–338, and Δ339–365, and the linker-insertion mutations *in*308, *in*321, *in*338, and *in*363 were introduced into the wt HIVgpt and each of the resultant plasmids was expressed in 293T cells. As expected, the Δ310–323 mutation had the effect of reducing virus particle production markedly (Fig. 5A, lane 5). Compared with the wt, the level of pelletable Gag proteins released by Δ310–323 was about 10 to 30% of the wt level (Fig. 5B). Virions released by the mutant *in*321 were also greatly reduced to a level of about 20 to 40% compared to the wt. Mutants *in*363 and Δ339–365 were both significantly impaired in virus assembly and release, which is consistent with previous reports stating that mutations in the C-terminus of CA result in disruption of HIV-1 virus assembly and release (Abdurahman et al., 2004; Melamed et al., 2004). Interestingly, a four-amino acid insertion at the C-terminus of MHR (*in*308) or mutations (*in*338 and Δ322–338) located in the region downstream of Δ310–323 but upstream of *in*363 had a less detrimental effect on virus particle assembly and release than Δ310–323 (Fig. 5A, lanes 3, 6, and 7). Notably, the PR-mediated proteolytic processing of all the mutants except *in*308 was impaired to a certain degree, particularly in the case of *in*321, Δ310–323, *in*363 and Δ339–365; all of them had the Gag precursor representing the major species in the medium samples (lanes 4, 5, 8, and 9). In addition to Pr55, p41, and p24/25, some $p24^{gag}$-associated products were detected, which may have resulted from mutation-induced aberrant cleavage since most of these products are not detectable when expressed in a PR-negative version or in the presence of a HIV-1 protease inhibitor (data not shown). Bands migrating slightly faster than $p24^{gag}$ and at positions corresponding to CA with small deletions were detected in mutants Δ310–323, Δ322–338, and Δ339–365. In contrast to the wt and other mutants, the mutant *in*363, with a substitution of three amino acid residues at the extreme C-terminus of CA, showed a relatively higher level of cell-associated p25, presumably due to a block in the cleavage at CA-SP1 (Fig. 5A, lane 17). Consistent with this finding, a mutant with a substitution mutation at the last amino acid residue of HIV-1 CA, and in which the CA-SP1 represents the major CA

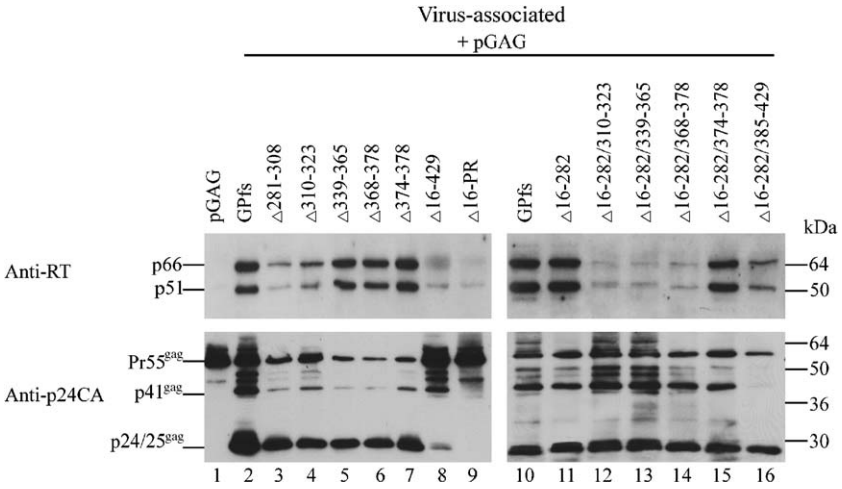

Fig. 2. Processing and release of HIV-1Gag and Pol proteins from cells coexpressing $Pr55^{gag}$ and $Pr160^{gag-pol}$. 293T cells were transfected with 10 μg of pGAG alone or together with 1 μg of the designated plasmid. At 48 h posttransfection, culture supernatants and cells were collected and prepared for protein analysis as described in Materials and methods. Supernatant samples corresponding to 50% of the total samples were fractionated by SDS-PAGE (10%) and electroblotted onto a nitrocellulose filter. HIV-1Gag and Pol proteins were probed with a mouse anti-$p24^{gag}$ and a mouse anti-HIV-1-RT monoclonal antibody, respectively. The positions of the molecular size markers are shown on the right and those of HIV-1Gag proteins Pr55, p41, p24/25, and RT proteins p66 and p51 are indicated on the left.

cleavage product, has been reported recently (Melamed et al., 2004).

Discussion

In this study, the ability of each of the HIV-1Gag–Pol deletion mutants to be incorporated into virions was judged by the level of virus-associated Gag–Pol protein. By coexpressing the wt or each of the PR-intact Gag–Pol mutants with pGAG at a DNA ratio of 10:1, mature virus-associated $p24^{gag}$ or RT could be detected readily in the cotransfectant culture medium. Exceptions to this rule were mutants Δ16–429 and Δ16-PR, which showed a poor Gag processing profile. This suggests that most Gag–Pol mutants could be incorporated into virions to a certain degree. We found that the experimental results were more reproducible and constant when the ratio of cotransfected Gag and Gag–Pol expression plasmid DNA was kept at 10:1 rather than at 20:1. Without coexpression with $Pr55^{gag}$, HIV-1 RT or Gag–Pol could not be detected in the medium samples, suggesting that the Gag–Pol is virus-associated (data not shown). The inability of both Δ16–429 and Δ16-PR to process Gag particles may be due in part to defective PR activity, as the level of intracellular $p24^{gag}$ produced by the two constructs was also markedly lower than those of the other Gag–Pol mutants (data not shown). This result is consistent with a previous report, which found that removal of the sequence upstream of the HIV-1 PR significantly affects PR activity (Zybarth and Carter, 1995).

When expressed in a PR-defective version, HIV-1 Gag–Pol precursors (Δ310–323) with 14 amino acid residues ($CA_{178–191}$) removed directly C-terminal to the MHR, were found to be severely defective in particle incorporation. In contrast to Δ310–323, both Δ339–365 and Δ281–308 (ΔMHR) showed no detrimental effect on Gag–Pol incorporation, and significant amounts of the two mutants were incorporated into virus particles at a level over 50% of that of wt Gag–Pol (Fig. 3). Although one previous study has shown that removal of the MHR in HIV-1 Gag–Pol significantly impairs the assembly of Gag–Pol into virus particles (Srinivasakumar et al., 1995), our previous and present studies have demonstrated that the Δ281–308 construct has only mild to moderate effect on the incorporation of Gag–Pol into virions (Chiu et al., 2002, 2004). Another group has reported that substitution mutations in the HIV-1 MHR have no significant effect on Gag–Pol incorporation (Mammano et al., 1994). It is unknown whether the three foreign amino acid residues Arg–Ile–Arg inserted in the deleted region of Δ281–308 have any positive effect on Gag–Pol incorporation. Alternatively, this discrepancy may be due to the different systems employed by the various research groups.

It has previously been demonstrated that chimeric Gag–Pol precursors in which the Moloney murine leukemia virus (MLV) MHR and the adjacent C-terminal CA sequences were replaced with their HIV-1 counterparts in a precise manner could still be incorporated into HIV-1 particles, suggesting that the MHR and adjacent CA in Gag–Pol is the region mainly responsible for the interaction with Gag (Huang and Martin, 1997). In accord with this report, we have shown that the Δ16–282 double mutations involving a removal of the C-terminus of CA (Δ16–282/339–365) or SP1 (Δ16–282/368–378) markedly inhibited the incorporation of Gag–Pol into virions (Fig. 2, lanes 13 and 14, and Fig. 3B, lanes 5 and 6). These HIV-1 Gag–Pol mutants contain large deletion mutations and therefore we could not exclude the possibility that the mutations may induce conformational changes in the encoded Gag–Pol and as a consequence interfered with the interactions between Gag–Pol and Gag. Nonetheless, we have defined a minimal sequence consisting of the MHR and the adjacent C-terminal CA-SP1 (Δ16–282/385–429) that can still direct the incorporation of Gag–Pol into virus particles with an efficiency over 70% of the wt level (Fig. 3B, lane 8). Our results presented here suggest that retention of an intact C-

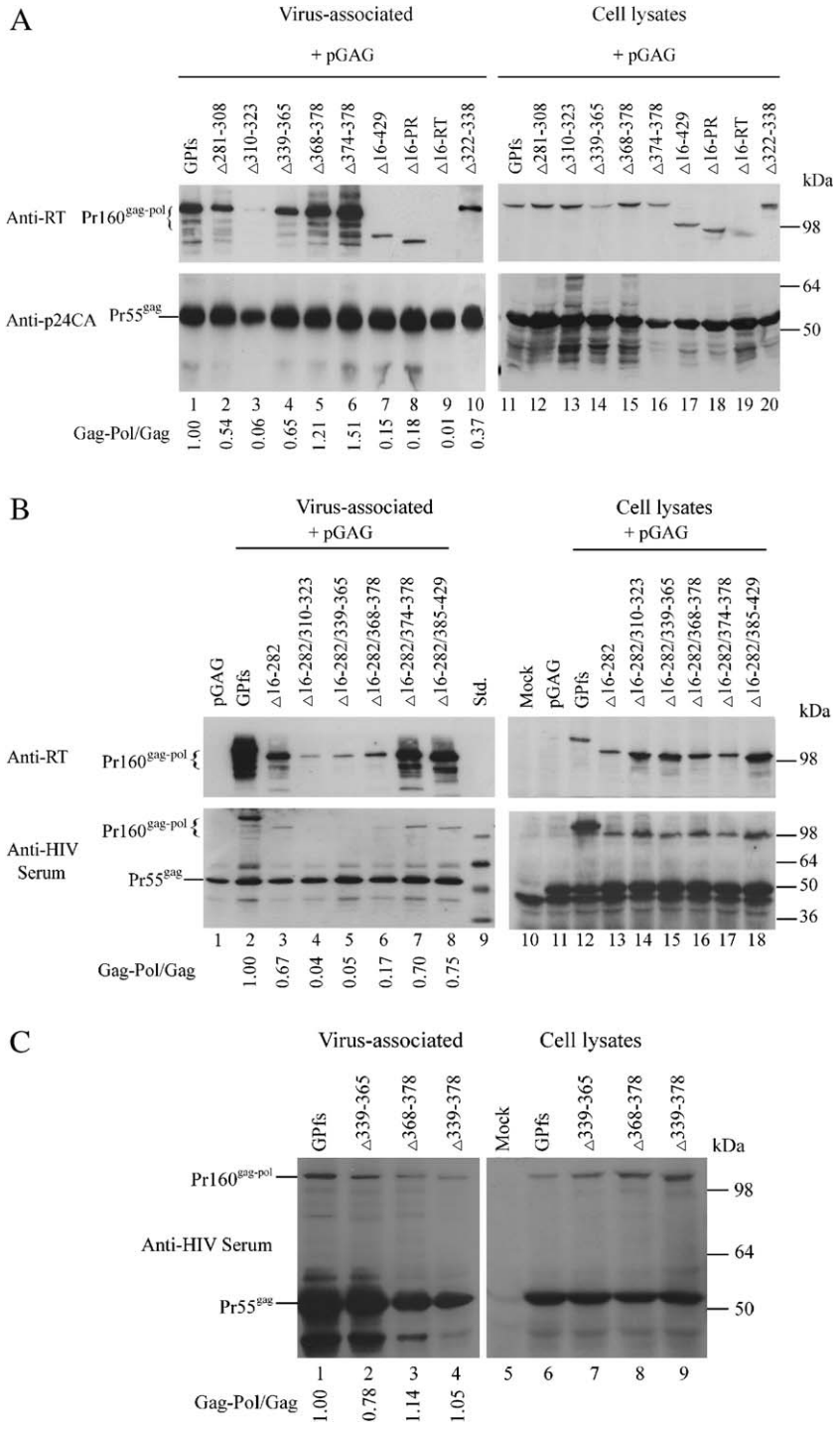

Fig. 3. Expression and incorporation of protease-defective (PR^-) Gag–Pol deletion mutants into virus particles. 293T cells were transfected with 10 μg of pGAG alone or together with 10 μg of the designated plasmids. All of the Gag–Pol constructs were expressed in a protease-inactivated version of GPfs. At 48 h posttransfection, culture supernatants were collected, filtered, and pelleted through 20% sucrose cushions. Viral pellets and cell lysates were prepared for protein analysis as described in Materials and methods. Membrane-bound HIV-1 proteins were probed with a mouse anti-HIV-RT monoclonal antibody or an HIV-positive human serum, followed by a goat anti-mouse or anti-human HRP-conjugated antibody as secondary antibody, respectively. Positions of molecular size markers (Std.) and those of the HIV-1$Pr55^{gag}$ and $Pr160^{gag–pol}$ are indicated. Levels of HIV-1Gag protein Pr55 and virus-associated Gag–Pol in each sample were quantified by scanning the density of the various bands on the immunoblot. The ratios of total Gag–Pol versus Gag protein levels were calculated for each of the samples, and normalized to that of wt GPfs in parallel experiments. Note that the immunoblots presented here were overexposed intentionally to allow visualization of the individual $Pr160^{gag–pol}$ mutants. Similar results were observed in a number of repeat and independent experiments.

terminus to CA and the adjacent SP1 is crucial for Gag–Pol incorporation when most of the N-terminal *gag* sequence upstream of the MHR has been removed.

With respect to the process of HIV-1 Gag assembly, the capsid C-terminal domain (CTD) including the MHR and the adjacent downstream sequence is thought to be crucial to Gag

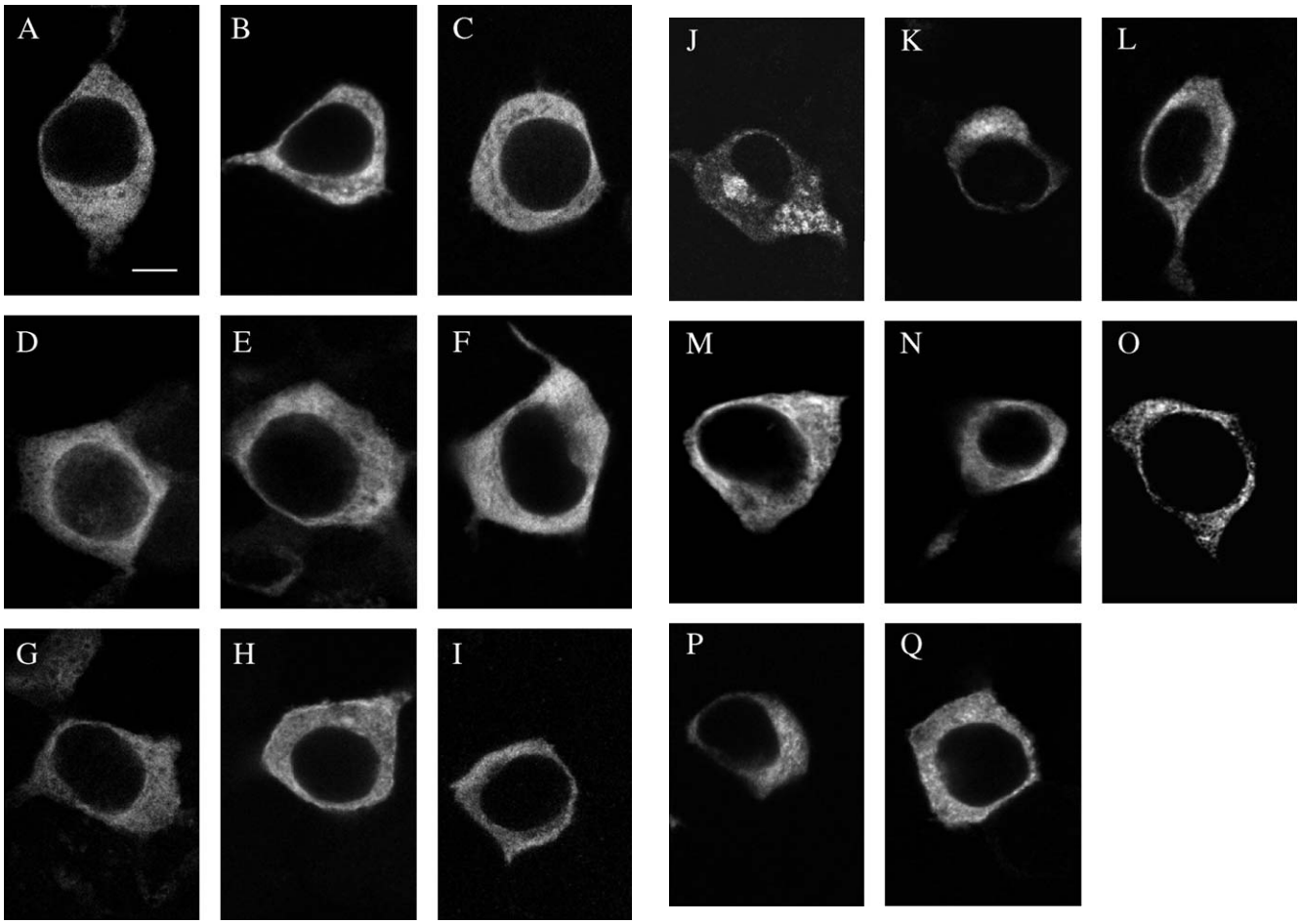

Fig. 4. Detection of HIV-1Pr160$^{gag–pol}$ proteins in 293T cells by indirect immunofluorescence. 293T cells grown on coverslips were transfected with the PR-defective version of wt GPfs (A) and mutants Δ281–308 (B), Δ310–323 (C), Δ322–338 (D), Δ339–365 (E), Δ368–378 (F), Δ374–378 (G), Δ385–429 (H), Δ16–429 (I), Δ16–PR (J), Δ16–RT (K), Δ16–282 (L), Δ16–282/310–323 (M), Δ16–282/339–365 (N), Δ16–282/368–378 (O), Δ16–282/374–378 (P), Δ16–282/385–429(Q). At 48 h posttransfection, cells were fixed and permeabilized for immunofluorescence assays as described in Materials and methods. The primary antibody was a mouse anti-HIV-RT monoclonal antibody (1:500), and the secondary antibody was a 1:100 dilution of a rhodamine-conjugated rabbit anti-mouse antibody. Data shown represent the most prevalent phenotypes. Mock-transfected 293T cells and cells not exposed to the primary antibody yielded no signals (data not shown). Scale bar in panel A represents 10 μm.

oligomerization and virus assembly and has been referred to as the dimerization domain. The dimerization domain contains four putative α-helix followed by a structurally disordered segment composed of the last 12 residues (Gamble et al., 1997; Momany et al., 1996; Worthylake et al., 1999). Crystallography studies have suggested the CTD (CA residues 146–231) dimerizes by parallel packing of the putative α-helix 2 (corresponding CA residues 179–192) across the dimer interface (Gamble et al., 1997). Thus, the Δ310–323 mutant, because of the removal of the CA residues 178–191, may have a disrupted Gag–Gag interaction due to removal of most of the α-helix sequence. In agreement with this observation, substitution mutations in CA residues W184 and M185, which have been removed in the mutant Δ310–323, have been reported to significantly reduce production of virus particles (von Schwedler et al., 2003). Crystallography studies of the HIV-1 CA suggest that both W184 and M185 are buried in the core of a symmetrical dimer interface, and alanine substitutions at either position block CA dimerization in vitro (Gamble et al., 1997; Ganser-Pornillos et al., 2004). In addition, we have very recently found that an alanine substitution at Q179 or K182 abolished virus assembly (unpublished results). Thus, the region immediately C-terminal to the MHR may be functionally involved in the process of Gag multimerization. The assembly defect caused by mutation Δ339–365 or *in*363 is not surprising, since a number of more recent studies have shown that substitutions or deletions of the last 11 conserved residues of CA, 354-VGGPGHLARVL-364, can cause a drastic reduction in the level of virus production, possibly due to impaired Gag multimerization (Abdurahman et al., 2004; Liang et al., 2003; Melamed et al., 2004; von Schwedler et al., 2003). That the Gag–Pol encoded by Δ339–365 is capable of being rescued into Gag particles suggests that mutations at the C terminus of CA do not disrupt the interactions of Gag–Pol with Pr55^{gag} to any great extent. Thus, Gag or Gag–Pol mutants containing mutations blocking virus assembly or Gag–Pol incorporation ought to still interact with the wt Gag. In agreement with this proposal, HIV-1 Gag or Gag–Pol carrying substitution mutations in the MHR show severe impairment of virus assembly, but have been shown to

Table 2
Summary of the relative efficiency of Gag–Pol incorporation and the intracellular immunofluorescence staining profiles of Gag–Pol mutants

Constructs	Virus-associated Gag–Pol [a]	Gag–Pol enriched in perinuclear areas [b]
Wild-type	++++	−
Δ281–308	+++	−
Δ310–323	−	−
Δ322–338	++	−
Δ339–365	+++	−
Δ368–378	++++	−
Δ374–378	++++	−
Δ339–378	++++	ND
Δ385–429	++++	−
Δ16–282	+++	−
Δ16–429	+	−
Δ16-PR	+	+++
Δ16-RT	−	+++
Δ16–282/310–323	−	−
Δ16–282/339–365	−	−
Δ16–282/368–378	+	−
Δ16–282/374–378	+++	−
Δ16–282/385–429	+++	−

[a] The ability of the Gag–Pol mutants to be incorporated into virus particles is indicated: ++++, incorporation efficiency comparable to wt (≥80% of wt); +++, efficiency about 50–70% of wt; ++, efficiency about 30–50% of wt; +, efficiency about 10–20% of wt; −, efficiency below 10% of wt.

[b] '+++' indicates over 90% of transfected 293T cells show fluorescence staining asymmetrically enriched in the perinuclear area. '−' Indicates a wild-type Gag–Pol distribution pattern with a heterogeneous cytoplasmic staining and a perinuclear ring; 'ND', not done.

retain the ability to interact with the wild-type Gag and be rescued into virus particles (Mammano et al., 1994). The detrimental effect of the Δ310–323 mutation on both virus assembly and Gag–Pol incorporation strongly suggests that the domain immediately C-terminal to the MHR is required to achieve efficient Gag/Gag or Gag/Gag–Pol interactions, which are essential for virus assembly and for incorporation of Gag–Pol into virions.

One recent study has demonstrated that HIV-1 Pol with a V5 tag can be incorporated into virus particles at a efficiency about 70% of that of wild-type Gag–Pol (Cen et al., 2004). Our present and previous studies indicate that HIV-1 Gag–Pol lacking the coding sequence upstream of the HIV-1 Pol or $p6^{pol}$ can still be incorporated into virions, albeit at a relatively lower efficiency (Fig. 3A, lanes 7 and 8). It is not known whether the retained N-terminal fourteen MA residues and the foreign residues inserted within the deleted region of our Gag–Pol constructs have any effect on the entry of the encoded Gag–Pol into virions. In the case of human foamy virus, the *pol* gene is expressed as Pro–Pol from separately spliced mRNA, and the Pol products are naturally incorporated into virions without the formation of Gag–Pol (Yu et al., 1996). Moreover, free MLV Pol has been shown to be capable of being incorporated into virions when MLV Pol and MLV Gag are coexpressed from separate plasmids (Buchschacher et al., 1999). These results suggest that retroviral Pol may contain undefined signals that are capable of interacting with Gag. Indeed, we have found that HIV-1 Pol truncated at IN can be incorporated into virions more efficiently than Δ16–429 (data not shown). Further studies are required to test whether the HIV-1 RT can specifically interact with Gag.

Indirect immunofluorescence studies suggested that Δ16-PR and Δ16-RT were both localized asymmetrically in the perinuclear area, which may account partly for their severe defect in being packaged into virus particles. However, most of the other mutants that were significantly defective in particle incorporation showed a subcellular localization pattern similar to that of wt. This suggests that cellular localization may not be the primary determinant of Gag–Pol incorporation into virus particles. At this point, we are unable to exclude the possibility that there are minor effects of the mutations on the subcellular localization of Gag–Pol as visualized by immunofluorescence that may be masked when using the 293T cell overexpression system. It has been demonstrated that the N-terminal myristylation signal required for both Gag plasma membrane targeting and virus assembly can be removed from Gag–Pol without impairing incorporation into virus particles (Chiu et al., 2002; Park and Morrow, 1992; Smith et al., 1993). One recent study has shown that the newly synthesized HIV-1 Gag–Pol colocalizes very rapidly with $Pr55^{gag}$ into detergent-resistant membrane (Halwani et al., 2003) suggesting that Gag may mediate the transport of Gag–Pol via Gag/Gag–Pol interactions. Collectively, these results suggest that the ability of each of the Gag–Pol deletions to be incorporated depends largely on how well the Gag–Pol mutant can interact with the coexpressed $Pr55^{gag}$. That both Δ16-PR and Δ16-RT tend to localize around perinuclear area suggests that removal of the transframe domain $p6^{pol}$ could significantly affect the subcellular distribution of Gag–Pol in the absence of the upstream Gag domain. It can be speculated that the karyophilic properties of IN, which may be exposed after the sequence upstream of PR has been removed, contribute to the perinuclear localization pattern exhibited by Δ16-PR and Δ16-RT. Experiments are in progress to test this possibility.

Materials and methods

Plasmid construction

To construct the Gag–Pol deletion mutants, a series of *Bam*HI sites were introduced in-frame into HIV-1 proviral positions at nt 836, 1751, 1803, 1884, 1907, 1923, 2076, and 2249 by PCR-mediated mutagenesis using HIVgpt (Page et al., 1990) or an HIV-1 Gag–Pol frameshift mutant, GPfs (Chiu et al., 2002) as template. *Bam*HI-created mutations at nt 1751, 1803, and 1884 are designated *in*321, *in*338, and *in*363, respectively, with the numbering referred to the Gag initiation methionine residue. The construct *in*308 was engineered by cutting viral DNA with *Hin*dIII (nt. 1712), filling-in and inserting an 8-mer *Bam*HI linker, which resulted in the insertion of four amino acid residues (Ala–Arg–Ile–Arg). DNA fragments spanning the above mutations were introduced individually into plasmid pBRHIV831–5786, which was constructed by cloning the HXB2 *Cla*I-831 to *Sal*I-5786 fragment into pBR322 vector. Replacement of the fragment *Cla*I-831 to *Bam*HI-1751 with the fragment Cla1-831 to

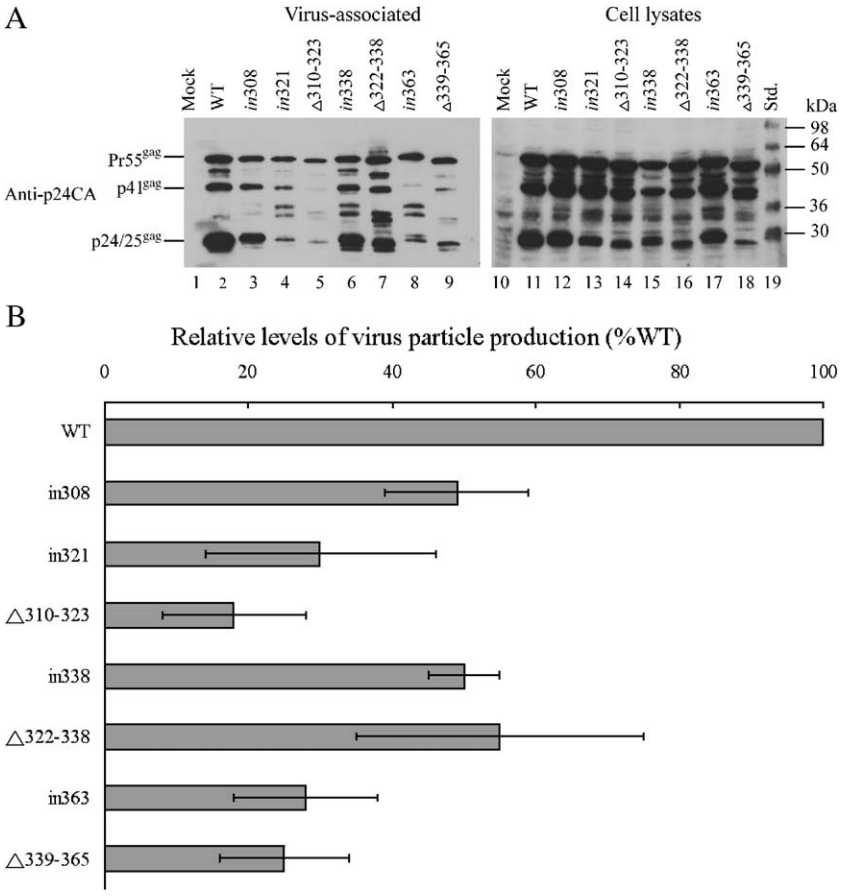

Fig. 5. Expression and release of wt and mutant Gag proteins. (A) 293T cells were transfected with wt HIVgpt and mutant plasmids. Two days after transfection, the cells and supernatants were collected for protein analysis as described in Materials and methods. Samples were fractionated by SDS-PAGE (10%) and then subjected to immunoblot analysis with an anti-p24^{gag} antibody as described in the legend to Fig. 2. The positions of molecular size markers (Std.) and those of HIV-1Gag proteins Pr55, p41, and p24/25 are indicated. (B) Relative levels of virus particle production. Gag proteins from medium were quantified by scanning the mutant and wt p24^{gag}-associated band densities from the immunoblots. The total arbitrary densitometer units of each mutant were normalized to that of the wt in parallel experiments. Values of the ratios indicate the relative levels of virus particles released. Error bars indicate the standard deviation.

*Bam*HI-1715 yielded the construct Δ310–323. By analogy, recombination among the other *Bam*HI linker-inserted mutants yielded deletion constructs Δ322–338, Δ339–365, Δ368–378, Δ339–378, Δ374–378, Δ385–429 and Δ16-PR. The *Cla*I-831 to *Sal*I-5786 fragments containing each of the deletion mutations were ligated into HIVgpt digested with *Cla*I and *Sal*I. Each of the mutant constructs was analyzed by restriction enzyme digestion and confirmed by DNA sequencing. The other Gag–Pol deletion constructs Δ16–282, Δ281–308, Δ16–429, Δ16-RT, and HIV-1 Pr55^{gag}-expression plasmid pGAG have been described previously (Chiu et al., 2002). The juncture sequences and altered amino acid residue for the Gag and Gag–Pol constructs are shown in Table 1.

Cell culture and transfection

293T cells were maintained in DMEM supplemented with 10% fetal calf serum. Confluent 293T cells were trypsinized, split 1:10 and seeded onto 10-cm plates 24 h before transfections. For each construct, 293T cells were transfected with 20 μg of plasmid DNA by the calcium phosphate precipitation method (Graham and van der Eb, 1973) with the addition of 50 μm chloroquine to enhance transfection efficiency. When the wild-type (wt) or mutant GPfs were cotransfected with pGAG, 1 μg of each construct and 10 μg of pGAG were used together with the addition of 9 μg pBlueScript SK, to give a final 20 μg plasmid DNA. For cotransfection of PR-defective (PR$^-$) GPfs constructs with pGAG, 10 μg of each construct and 10 μg of pGAG were used. Culture media and cells were harvested for protein analysis at 48 h posttransfection.

Western immunoblot analysis

Culture medium from transfected 293T cells was filtered through 0.45-μm pore-size filters; this was followed by centrifugation at 4 °C through 2 ml 20% sucrose in TSE (10 mM Tris–HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA) containing 0.1 mM phenylmethylsulfonyl fluoride (PMSF) for 40 min at 274,000×*g* (SW41 rotor at 40,000 rpm). Viral pellets then were suspended in IPB (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, 0.02% sodium azide)

containing 0.1 mM PMSF. The cells were rinsed with ice-cold phosphate-buffered saline (PBS), scraped from the plates, collected in 1 ml PBS and pelleted at 2500 rpm for 5 min. The cell pellets were resuspended in 250 μl IPB containing 0.1 mM PMSF, and then subjected to microcentrifugation at 4 °C for 15 min at 13,700×*g* (14,000 rpm) to remove cell debris. Supernatant and cell samples were mixed with equal volumes of 2× sample buffer (12.5 mM Tris–HCl, pH 6.8, 2% SDS, 20% glycerol, 0.25% bromophenol blue) and β-mercaptoethanol (5%), and boiled for 5 min. The samples were subjected to SDS-PAGE and electroblotted onto nitrocellulose membranes. The membranes were blocked with 3% gelatin in Tris-buffered saline containing 0.05% Tween 20 (TBST), followed by incubation with the primary antibody in 1% gelatin-TBST for 1 h on a rocking platform at room temperature. The membranes were then washed three times for 10 min each with TBST, and rocked for 30 min with the secondary antibody in 1% gelatin-TBST. The blots were again washed three times in TBST for 10 min each, and the membrane-bound antibody-conjugated enzyme activity was detected by an enhanced chemiluminescence (ECL) detection system or by a colorimetric method. For detection of HIV-1 Gag proteins, we used anti-p24^{gag} (a mouse hybridoma clone 183-H12-5C) monoclonal antibody at a 1:5,000 dilution from ascites. To detect HIV-1 RT or Gag–Pol deletion mutants, a mouse monoclonal antibody against HIV-1 RT or an HIV-positive human serum was used at 1:10,000 dilution as the primary antibody. The secondary antibody was either alkaline phosphatase conjugated horse anti-mouse IgG (Vector Laboratories), HRP-conjugated goat anti-human antibody, or HRP-conjugated rabbit anti-mouse antibody (Pierce). Procedures for detection of alkaline phosphatase and HRP activity followed the manufacturers' protocols.

Indirect immunofluorescence

The protocol for immunofluorescence was as previously described (Wang et al., 1998). Briefly, confluent 293T cells were split 1:80 and seeded onto coverslips 24 h before transfection. Two days after transfection, the cells were fixed at 4 °C for 20 min with ice-cold PBS containing 3.7% formaldehyde. They were then washed once with PBS and once with DMEM containing 10% heat-inactivated calf serum (DMEM/calf serum) and permeabilized at room temperature for 10 min in PBS containing 0.2% Triton X-100. Samples were incubated with the primary antibody for 1 h and secondary antibody for 30 min. Following each incubation, the samples were subjected to three 5- to 10-min washes with DMEM/calf serum. The primary antibody was a mouse anti-HIV-1 RT monoclonal antibody (Exalpha Biologicals Inc.) used at 1:500 dilution, and the secondary antibody was a rabbit anti-mouse rhodamine-conjugated antibody at 1:100 dilution (Cappel). After the last DMEM/calf serum wash, the coverslips were washed three times with PBS and mounted in 50% glycerol. Images were analyzed and photographs taken using an Olympus FV300 confocal microscope.

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